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Screening on the Presence of Plant Growth Regulators in High Biomass Forming Seaweeds from the Ionian Sea (Mediterranean Sea)

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Abstract: The use of seaweed as plant biostimulants is a solution for sustainable agriculture. The present study aims to quantify and compare the presence of plant growth regulators (PGRs) in four genetically labeled macroalgae growing in the Ionian Sea. Species were selected because they produce abundant biomass, disturbing ecological equilibrium and anthropic activities. We measured the content of gibberellic acid (GA_3), kinetin (KN), indoleacetic acid (IAA), abscisic acid (ABA) and indole butyric acid (IBA). The method applied was modified from the literature to obtain simultaneously different PGRs from seaweed biomass in a shorter period of time. Among results, it is notable that *Hypnea corona* Huisman et Petrocelli (*Rhodophyta*) showed higher GA_3 concentration, while in *Spyridia filamentosa* (Wulfen) Harvey (*Rhodophyta*), higher KN, IBA, IAA and ABA contents were recorded. The latter species displayed an interesting profile of PGRs, with an IAA value comparable with that reported in *Ascophyllum nodosum* (Linnaeus) Le Jolis (*Ochrophyta*), which is currently used as a source of plant biostimulants in agriculture. Macroalgae thrive abundantly in nutrient-rich environments, such as anthropized coastal areas affecting human economic activities. Consequently, environmental agencies are forced to dredge algal thalli and discard them as waste. Any use of unwanted biomass as an economic product is highly desirable in the perspective of ecosustainable development.

Keywords: algal biomass; plant biostimulants; HPLC; plant growth regulators; seaweed extracts; sustainable agriculture

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Citation: Spagnuolo, D.; Russo, V.; Manghisi, A.; Di Martino, A.; Morabito, M.; Genovese, G.; Trifilò, P. Screening on the Presence of Plant Growth Regulators in High Biomass Forming Seaweeds from the Ionian Sea (Mediterranean Sea). *Sustainability* **2022**, *14*, 3914. https://doi.org/10.3390/su14073914

Academic Editors: Carmelo M. Musarella, Ana Cano Ortiz, Ricardo Quinto-Canas and Antonio Jesus Mendoza-Fernández

Received: 9 February 2022
Accepted: 23 March 2022
Published: 25 March 2022

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1. Introduction

Increasing drought events, as a consequence of climate change, are causing on a global scale relevant loss in crop yield [1]. Water availability strongly affects plant productivity [2]. Nevertheless, as a likely effect of climate change, an increase in the frequency of extreme events, such as heatwaves, flooding, hurricanes, etc., negatively impacts plant resilience ability, exposing vegetation to higher crop disease and, as a consequence, exacerbating yield reductions [3,4]. Based on alarming forcasts on global warming [5], improving, as much as possible, the resistance and resilience ability as well as the yield of crop plants in such "new" growth conditions is a priority. This challenging task is further needed to meet food demand due to population growth [6]. Optimal mineral nutrition increases plant resilience to different biotic and abiotic stresses, as well as food quality [7]. Nevertheless, chemical fertilization causes high economic and ecological costs [8]. The common practice of improving crop productivity and/or food quality, by using synthetic plant growth

regulators (PGRs) in addition increasing management costs, could be toxic for plants and animals, including humans [9–11].

Plant biostimulants are products that respond to the requirement for agriculture products that are less dependent on synthetic chemicals and at the same time able to provide greater yields and mitigate the effects of climate change, stimulating plant nutrition processes, the tolerance to abiotic stress and crop quality [12–14].

On this view, the use of seaweed is actually a solution for sustainable agriculture because it combines the need to use low-cost but good fertilizers (i.e., it contains minerals) with a source of plant biostimulants. Furthermore, it has the added value of providing a solution to the disposal of unwanted seaweed biomass, which may occur especially in eutrophic environments [15–17].

Seaweeds have been used as an organic fertilizer since ancient times [18]. However, since the 1960s, the seaweed industry has received renewed interest because of an increasing number of studies demonstrating the positive effects of using seaweed extracts on crop productivity and food quality [19].

Seaweeds are mainly applied in organic agriculture due to their biodegradable, non-toxic, non-polluting and non-hazardous effects relative to human and animals [20]. In this regard, extracts obtained by macroalgae represent 30% of the market of plant biostimulants in 2013, 40% of which is absorbed by the European market [21]. Early studies aimed to identify mineral content of seaweed extracts [22,23]. By contrast, in the most recent years, greater attention has been given to PGR contents and other organic compounds frequently present in these natural plant biostimulants.

Macroalgae produce huge biomass in nutrient rich environments, which often are dredged and discarded as waste that do not affect human activities. Such biomass could be a promising source of PGRs in the perspective of eco-sustainable development.

A high and increasing number of studies on the physiological roles of phytohormones in terrestrial plants improve our understanding of their effects and interactions on plant growth and productivity. Conversely, the same knowledge is lacking for seaweeds [24]. This gap may negatively impact the advantages of using seaweed extracts on crops. On this view, verifying and quantifying the presence of plant biostimulant products in the algae are prerequisites to any project related to their physiological involvement.

The present study aims to quantify and compare the presence of some PGRs in four different macroalgae growing in the Ionian Sea (Taranto, Italy), i.e., two *Rhodophyta* (*Spyridia filamentosa* (Wulfen) Harvey and *Hypnea corona* Huisman et Petrocelli) and two *Chlorophyta* (*Chaetomorpha linum* (O.F. Müller) Kützing and *Ulva lacinulata* (Kützing) Wittrock). The four algal species were selected because they produce abundant biomass in the collection site, disturbing both ecological equilibrium and anthropic economic activities.

In detail, we measured the content of gibberellic acid (GA_3), kinetin (KN) indoleacetic acid (IAA), abscisic acid (ABA) and indole butyric acid (IBA) as the main representatives of PGRs as a screening prerequisite for further tests to evaluate their agronomic effects.

2. Materials and Methods

2.1. Collection of Algae

Samples of *Spyridia filamentosa* (Wulfen) Harvey, *Hypnea corona* Huisman et Petrocelli (*Rhodophyta*), *Chaetomorpha linum* (O.F. Müller) Kützing and *Ulva lacinulata* (Kützing) Wittrock (*Chlorophyta*) were collected from Taranto (Italy, Ionian Sea) (Table 1).

Species names and phylum attributions are in accordance with algaebase.org [25]. All species used in the present investigation have isomorphic life cycles, with alternating gametophytic and sporophytic phases, which thrive in mixed populations and are distinguishable only by fine reproductive aspects by trained experts. In the perspective of potential economic exploitation, we decided to use natural populations and to process mixed haploid and diploid batches.

Table 1. List of the algal samples used in this study.

	Species	Voucher ID
Rhodophyta	*Hypnea corona* Huisman et Petrocelli	PhL705
	Spyridia filamentosa (Wulfen) Harvey	PhL706
Chlorophyta	*Chaetomorpha linum* (O.F. Müller) Kützing	PhL707
	Ulva lacinulata (Kützing) Wittrock	PhL708

After collection, samples were immediately washed with seawater to remove possible debris, adhering sand particles and epiphytes and then transported to the laboratory in plastic bags at low temperatures and washed with tap water to remove surface salt. From each sample, a portion was dried in silica gel and stored at −20 °C for DNA barcoding identification, and the remaining portions were dried for 72 h in an oven at 65 °C. Then, the samples were powdered by an electric grinder stored in plastic bags at 4 °C until they were analyzed.

DNA barcoding identification was performed according to protocols described in Miladi et al. [26] and Manghisi et al. [27]. Selected barcodes were COI-5P for *Rhodophyta*, tufA for *U. lacinulata* and LSU D2/D3 for *C. linum* [28]. DNA sequencing reactions were performed by an external company (Macrogen Europe, Amsterdam, The Netherlands). Forward and reverse sequence reads were assembled with the software ChromasPro (v. 1.41, Technelysium Pty Ltd., South Brisbane, QLD, Australia), and species attributions were performed by the identification engine in BOLD Systems (www.boldsystems.org, accessed on 6 January 2022) and the BLAST tool at the National Center for Biotechnology Information (Bethesda, MD, USA, blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 6 January 2022).

2.2. Preparation of Standard Solutions

Standard substances (purity > 98%) of gibberellic acid (GA_3), indoleacetic acid (IAA), abscisic acid (ABA) and indole butyric acid (IBA) were purchased from OlChemIm s.r.o. (Olomouc, Czech Republic). Kinetin (KN) was purchased from Sigma Aldrich (St. Louis, MO, USA). Analytical HPLC-grade methanol (MeOH) and glacial acetic acid were obtained from Merck (Darmstadt, Germany). Distilled water was deionized in an Elga Veolia Purelab ultra-pure water system (High Wycombe, UK).

The standard compounds were dissolved in MeOH:H_2O (50:50 v/v) at a stock concentration of 1000 µg/mL and stored at 4 °C. Working standard solutions were obtained by diluting them with MeOH: H_2O (50:50 v/v) prior to use. All solvents were ultrasonified for 30 min (Sonica, Soltec, Japan) before use.

2.3. Sample Preparation

An aliquot (0.5 g) of each powdered sample was infused in a 4 mL MeOH:H_2O solution (80:20 v/v) with 1 mmol/L of citric acid as the antioxidant. Solutions were sonicated for 15 min and placed in infusion for 3 days at 4 °C. The samples were transferred in 15 mL vials and centrifuged at 7000 rpm for 30 min at 4 °C. Then, the supernatants were collected and 1 mL of MeOH was added. After 1 h, the samples were centrifuged for an additional 15 min at 7000 rpm at 4 °C, filtered with 0.2 µm in diameter syringe filter and diluted with ultra-pure water (1:5). No purification steps were performed to speed up the protocol.

2.4. HPLC Set-Up

Chromatographic runs were carried out on a Beckman Coulter 126 binary pumps HPLC system with the detector Beckman Coulter 166 UV/VIS system (Brea, CA, USA). Karat 32 ver. 8.0 software was employed for instrument control and data acquisition. Data analyses were accomplished by in-house Octave script. Starlab scientific (Xi'an, China) XChroma universal-C18 column (5 µm, 120 Å, 4.6 × 250 mm) was used as the separation channel.

The mobile phase was composed of MeOH:H_2O (70:30, v/v), both acidified with acetic acid 0.5%, and the flow rate was 1.0 mL/min. The UV/Vis detector was set to 280 nm. The injection volume was 20 µL for each analysis using IDEX corp. Rheodyne 7125 valve (Lake Forest, CA, USA). All samples were analyzed in 3 repetitions. The results are presented as mean ± standard deviation.

2.5. Method Validation

The HPLC method was validated by the evaluation of the variation of retention times and peak area for analytes, performing calibration curves, limit of detection (LOD), limit of quantification (LOQ) and accuracy.

The analytical performances and the calibration curve are summarized in Table 2. The limit of detection (LOD), obtained by evaluation of signal to noise ratio, ranges between 5 µg/mL for GA_3 and 0.2 µg/mL for KN, IAA and ABA. The calibration curves showed a linear trend, and the reliability of measurements were confirmed by intra- and inter-day analysis, and the standard deviations are less than 2%. The retention time of the standards was 4.6, 5.3, 7.5, 9.2 and 12.3 min for GA_3, KN, IAA, ABA and IBA, respectively.

Table 2. Analytical performance data for major endogenetic plant growth regulators. R^2: correlation coefficient. LOD: limit of detection.

Analyte	Range (µg/mL)	Equations	R^2	LOD (µg/mL)	Degree of Freedom
GA_3	10–1000	y = 0.0433x + 0.0013	0.9896	5	6
KN	0.1–10	y = 3.9094x − 0.0008	0.9937	0.2	6
IAA	0.1–10	y = 0.5829x + 0.0006	0.9967	0.2	6
ABA	0.1–10	y = 1.8133x − 0.0004	0.9985	0.2	6
IBA	0.1–10	y = 0.4685x + 0.0007	0.9965	1.8	6

The standard solutions were found to be stable for months and stored at −25 °C; any variations on the value of response function were observed in the chromatogram recorded.

3. Results and Discussion

Seaweeds are known to produce plant growth regulators (PGRs), similarly to land plants [29]. Their effects include the response to various developmental and physiological processes and provide support to overcome abiotic and biotic stresses [30]. Recently, the attention of researchers pointed to the detection and quantification of different PGRs in seaweeds with the aim of agronomic applications [30].

The method applied in the present work was modified from Gupta et al. [31] in order to obtain simultaneously different PGRs from seaweed biomass but in a shorter period time. The separation of PGRs was performed by simplifying the extraction process, with a complete run performed in 18 min. The most significant modification was the lack of purification of the extracts in order to make the protocol faster and cheaper in the framework of applicative exploitation.

Overall, the *Rhodophyta* species analyzed in the present work showed a higher content of PGRs than the analyzed *Chlorophyta* (Table 3, HPLC chromatograms in Supplementary Materials). These data, however, cannot be drawn as a general conclusion that *Rhodophyta* as a whole have a higher content of PGRs than *Chlorophyta*, as the present results regard a limited taxonomic span. More research is needed to achieve a general framework.

In detail, *Hypnea corona* Huisman et Petrocelli showed higher GA_3 concentration, while in *Spyridia filamentosa* (Wulfen) Harvey, higher KN, IBA, IAA and ABA contents were recorded. The latter species displayed an interesting profile of PGRs, with an IAA value comparable with that reported in *Ascophyllum nodosum* (Linnaeus) Le Jolis (*Ochrophyta*) [32], which is currently used as a source of plant biostimulants in agriculture [33–36].

Table 3. Gibberellic acid (GA_3), kinetin (KN) indoleacetic acid (IAA), abscisic acid (ABA) and indole butyric acid (IBA) contents as estimated by HPLC-UV in extracts of the four investigated seaweed extracts. Values are presented as means of three measurements with standard deviations. LOD: limit of detection. LOQ: limit of quantification.

Species	GA_3 (µg/mL)	KN (µg/mL)	IAA (µg/mL)	ABA (µg/mL)	IBA (µg/mL)
Hypnea corona Huisman et Petrocelli	1038.00 ± 2.00	0.57 ± 0.07	6.70 ± 0.30	1.10 ± 0.40	LOQ
Spryridia filamentosa (Wulfen) Harvey	6.30 ± 0.10	1.70 ± 0.20	63.60 ± 0.50	8.40 ± 0.90	17.90 ± 0.00
Chaetomorpha linum (O.F. Müller) Kützing	5.40 ± 0.40	0.31 ± 0.05	LOD	0	LOQ
Ulva lacinulata (Kützing) Wittrock	0	0.48 ± 0.06	2.30 ± 0.10	0.72 ± 0.02	LOQ

In *Chaetomorpha linum* (O.F. Müller) Kützing, no IAA, IBA and ABA were present. *Ulva lacinulata* (Kützing) Wittrock showed a concentration of KN and ABA similar to *H. corona* but lower than *S. filamentosa* and no GA_3 content.

The presence of PGRs in seaweeds has been already reported in several studies [31,37,38]. However, the occurrence and the roles of these molecules on seaweeds physiology are still not clear [39–41].

Nevertheless, the literature data strongly suggest that PGRs in seaweeds are not a mere result of some metabolic processes but may have specific physiological relevance on their growth as a response to environmental stimuli [37]. In accordance, it has been shown that changes in PGR concentration occur in response to abiotic stress in different seaweed species [38,39,42]. Moreover, different studies highlighted similar roles of PGRs in terrestrial plants versus seaweeds species. As an example, IAA affects the embryo development of *Brassica juncea* (Linnaeus) Czernajew (*Magnoliophyta*) as well as of *Fucus distichus* Linnaeus (*Ochrophyta*) germlings [43,44]. Similarly to terrestrial plants, ethylene promoted chlorophyll degradation in *Ulva intestinalis* Linnaeus [45] and affected the maturation of reproductive structures in *Pterocladiella capillacea* (S.G. Gmelin) Bornet (*Rhodophyta*) [46]. ABA, a phytohormone generally associated to several stress responses in terrestrial plants [47,48], is involved in the coping oxidative stress of intertidal seaweed species [49]. Stirk et al. [50] recorded higher ABA content in *Ulva lactuca* Linnaeus (as *Ulva fasciata* Delile) versus *Dictyota humifusa* Hörnig, Schnetter et Coppejans (*Ochrophyta*), likely as a result of a stronger environmental stress. Brassinosteroided-mediated ABA synthesis occurred in response to thermal stress in brassicacean *Chorispora bungeana* Fischer et C.A. Mey (*Magnoliophyta*) as well as in green alga *Chlorella vulgaris* Beijerinck (*Chlorophyta*) [51,52]. Furthermore, in terrestrial plants and frequently but not exclusively in response to biotic and abiotic stresses, ABA plays a key role in different developmental processes, including seed germination, root and shoot development and photosynthesis inhibition [53–58]. Likewise, ABA impacted plant growth in *Laminaria* J.V. Lamouroux spp. (*Ochrophyta*), inhibited the growth of sporophyte, but it stimulated sorus formation in *Saccharina japonica* (Areschoug) C.E. Lane, C. Mayes, Druehl et G.W. Saunders (as *Laminaria japonica* Areschoug) and constrained the photosynthesis of *Fucus vesiculosus* Linnaeus embryos [59–61].

4. Conclusions

In the present study, PGRs were recorded in four macroalgal species belonging to *Rhodophyta* and *Chlorophyta*. Their physiological role was not investigated, which was out of the scope of our research. However, it could be speculated that PGRs in algae affect their growth and response to environmental factors, similarly to terrestrial plants in accordance with the literature (see above). Even if understanding the physiological functions of PGRs in algae is very interesting for fundamental physiological research, it is not essential for seaweed industrial exploitation, including the use of algal biomass as a source of plant biostimulants in the framework of sustainable agriculture. Further studies are needed to evaluate the effective applications of algal PGRs in agriculture, testing both protocols to produce algal plant biostimulants and application strategies on the growth of agronomic species in the field.

The novelty of the present research relies not only in reporting the presence of PGRs in algae as a whole but also on suggesting the use of unwanted biomasses and testing them for the presence of PGRs for the purpose of their exploitation in agriculture. Moreover, due to the large genetic and consequently metabolic diversity of macroalgae, the aim is to investigate more taxa as a source of PGRs from diverse geographical sites and add useful data in the knowledge of algal physiology.

Macroalgal identification at the species level is a complex task, which need the involvement of skilled taxonomists. In the perspective of industrial exploitation, we recommended that genetic labeling should be used. The official DNA barcode is a prompt and effective tool, which proved to be useful in applied research, e.g., [62–64].

Macroalgae thrive abundantly in nutrient rich environments, such as anthropized coastal areas. Such biomass affects human economic activities, disturbing navigation, aquaculture and tourism, as examples. As a consequence, environmental agencies are forced to dredge algal thalli and discard them as a waste. Any use of unwanted biomass as an economic product is highly desirable in the perspective of eco-sustainable development.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su14073914/s1, Figure S1: *Hypnea corona* Huisman et Petrocelli, HPLC chromatogram. Minutes reported in decimal divisions; Figure S2: *Spyridia filamentosa* (Wulfen) Harvey, HPLC chromatogram. Minutes reported in decimal divisions; Figure S3: *Chaetomorpha linum* (O.F. Müller) Kützing, HPLC chromatogram. Minutes reported in decimal divisions; Figure S4: *Ulva lacinulata* (Kützing) Wittrock, HPLC chromatogram. Minutes reported in decimal divisions.

Author Contributions: Conceptualization, D.S., V.R. and P.T.; methodology, D.S., V.R., A.M. and A.D.M.; writing—original draft preparation, D.S., V.R., A.M. and A.D.M.; writing—review and editing, M.M., G.G. and P.T.; funding acquisition, V.R., M.M., G.G. and P.T. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by South Agro srl (Ordinary Operating Funds 2020), FFABR2017-UniMe (Italian Ministry of University and Research), to M.M. and P.T., FFABR2019-UniMe (Italian Ministry of University and Research) to G.G.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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